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Akt interacts directly with Smad3 to regulate the sensitivity to TGF-β-induced apoptosis

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Transforming growth factor β (TGF-β) induces both apoptosis and cell-cycle arrest in some cell lines, but only growth arrest in others[1]. It is not clear how this differential response to TGF-β is specified. Smad proteins are critical mediators of TGF-β signalling. After stimulation by TGF-β, Smad2 and Smad3 become phosphorylated by the activated TGF-β receptor kinases, oligomerize with Smad4, translocate to the nucleus and regulate the expression of TGF-β target genes[1–5]. Here we report that the sensitivity to TGF-β-induced apoptosis is regulated by crosstalk between the Akt/PKB serine/threonine kinase and Smad3 through a mechanism that is independent of Akt kinase activity. Akt interacts directly [AU: OK?] with unphosphorylated Smad3 to sequester it outside the nucleus, preventing its phosphorylation and nuclear translocation. This results in inhibition of Smad3-mediated transcription and apoptosis. Furthermore, the ratio of Smad3 to Akt correlates with the sensitivity of cells to TGF-β-induced apoptosis. Alteration of this ratio changes the apoptotic, but not the growth-inhibitory, responses of cells to TGF-β. These findings identify an important determinant of sensitivity to TGF-β-induced apoptosis that involves crosstalk between the TGF-β and phosphatidylinositol-3-OH kinase (PI(3)K) pathways.

Although several downstream mediators of TGF-β-induced apoptosis have been reported[6–10], the intracellular signalling events that regulate this process have not been well defined. High levels of Smad3, but not Smad2, promote TGF-β-induced apoptosis, whereas insulin or insulin-like growth factor-1 (IRS-1) antagonizes this response both *in vivo* and *in vitro*[11–17]. Moreover, overexpression of either an activated form of PI(3)K, IRS-1, or Akt in liver cells mimics the anti-apoptotic effect of insulin[17–19]. Thus, activation of Akt seems to be necessary for the protection of liver cells from TGF-β-induced apoptosis, but the mechanism involved has not been identified. In a systematic screening for Akt-associated proteins, Smad3 was identified as a binding partner (Remy *et. al*, accompanying paper [AU: **will include this ref. as a note added in proof.**]). We therefore investigated the possibility that Akt may exhibit crosstalk with Smad3 to regulate the sensitivity to TGF-β-induced apoptosis.

In 293T cells co-transfected with various Flag-tagged Smad proteins and haemagglutinin (HA)-tagged Akt, Akt co-precipitated with Smad3, but not with the highly homologous Smad2, Smad4, the BMP-specific Smad1 and Smad5 or inhibitory Smad6 and Smad7 (Fig. 1a). This interaction was mediated by the linker and MH2 domains of Smad3 (S3LC: residues 230–425; Fig. 1b) and the carboxy-terminal half of Akt (glutathione *S*-transferase (GST)–Akt-C: residues 230–425; Fig. 1c). Recombinant Smad3 and an Akt fragment purified from *Escherichia coli* also bound to each other *in vitro* (Fig. 1c), indicating that the interaction is direct.

As Akt and Smad3 are mediators of insulin and TGF-β signalling, respectively, we asked whether endogenous Akt and Smad3 also interact and whether this interaction is regulated by TGF-β and insulin. As shown in Fig. 1d, endogenous Akt associated with Smad3 in the absence of exogenously added growth factors. Treatment with TGF-β disrupted this interaction, whereas stimulation with insulin slightly enhanced it. Interestingly, treatment of cells with both TGF-β and insulin completely prevented the disruption of the Akt–Smad3 complex by TGF-β. This differs quantitatively from the observation by Remy *et al.* (accompanying paper) that insulin only partially blocked the disruptive effect of TGF-β. This may be caused by differences in the duration of insulin treatment and other experimental conditions. Taken together, our results showed that insulin and TGF-β exert opposing effects on the Akt–Smad3 interaction.

TGF-β induces phosphorylation of Smad3 at C-terminal serine residues[1]. As Smad3 interacts with Akt only in the absence of TGF-β, phosphorylation of Smad3 may negatively regulate this interaction. Indeed, a mutant of Smad3 lacking the phosphorylation sites (3A) bound to Akt as well as, if not better than, wild-type Smad3 (Fig. 1e). In contrast, the 3D mutant that simulated phosphorylation exhibited a reduced binding to Akt, indicating that phosphorylation of Smad3 weakens this interaction. Consistently, no significant level of phospho-Smad3 was detected in the Smad3–Akt complex, either in the absence or presence of TGF-β (Fig. 1f).

Activation of Akt by insulin requires phosphorylation on Ser 473 and Thr 308 (ref. 20). As insulin enhances the interaction of Akt and Smad3, we asked whether this enhancement is caused by the phosphorylation of Akt. Mutations in Akt that remove the two phosphoryla-

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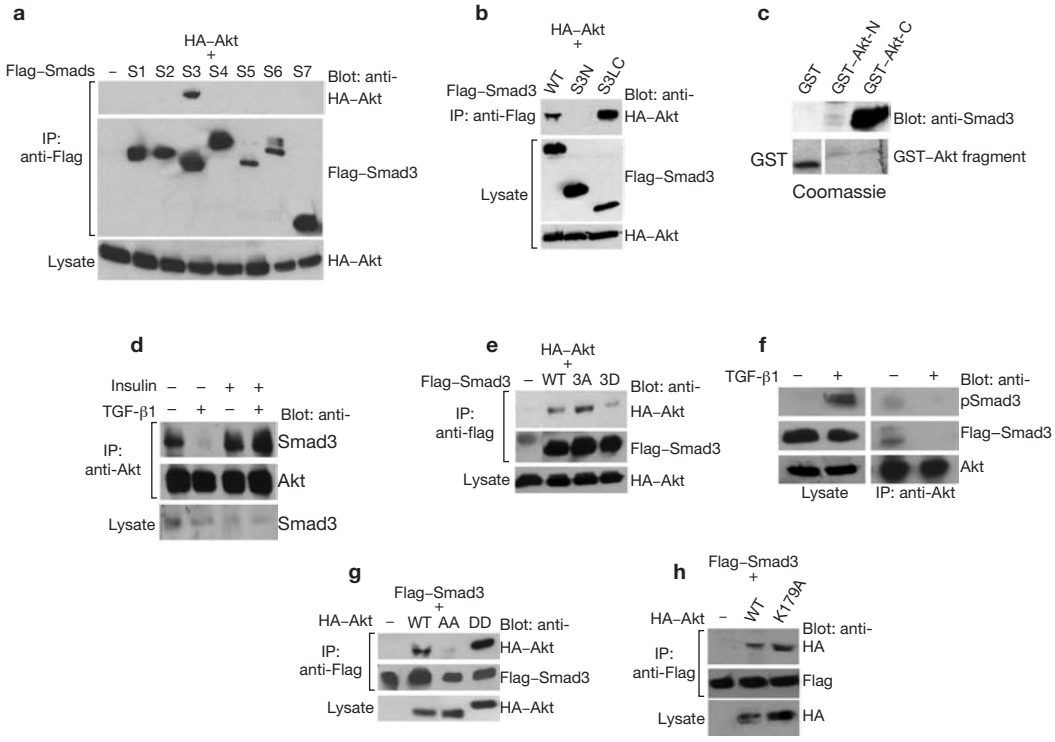

Figure 1 Akt interacts with Smad3. (**a**) Flag-tagged Smads were cotransfected with HA–Akt into 293T cells and isolated by immunoprecipitation with anti-Flag–agarose. Smad-bound Akt was detected by western blotting with anti-HA. Immunoprecipitates were blotted with anti-Flag to control for Smad expression. Cell lysates were blotted with anti-HA to control for HA–Akt expression. S1–S7: Smad1–Smad7. (**b**) Akt interacts with the MH2 and linker region of Smad3. Interaction of HA–Akt and Flag-tagged Smad3 fragments was determined as described in **a**. S3N: MH1 domain of Smad3; S3LC: linker and MH2 domains of Smad3. (**c**) Smad3 interacts directly with Akt. Bacterially purified Smad3 was incubated with immobilized GST–Akt fragments. Akt-bound Smad3 was eluted with glutathione and detected by western blotting with anti-Smad3. Levels of GST–Akt-N (residues 1–240) and GST–Akt-C (residues 241–480) were determined by Coommassie staining (bottom). (**d**) Interaction of endogenous Akt and Smad3 is regulated by TGF-β and insulin. Lysates from untreated or treated Ba/F3 cells were subjected to immunopreciptation with anti-Akt before western blotting with anti-Smad3 (top) or anti-Akt (middle). **[AU: Please also describe bottom 'Lysate' panel.]** (**e**) Phosphorylation of Smad3 decreases the Akt–Smad3 interaction. Experiments were performed as described in **a**. (**f**) Akt binds to unphosphorylated Smad3. TGF-β-treated Ba/F3/F Smad3 cells were subjected to immunoprecipitation with anti-Akt before western blotting with anti-phospho-Smad3 to measure Akt-bound phospho-Smad3, and with anti-Flag or anti-Akt to control for the level of Akt-associated Smad3 or Akt. Cell lysates were immunoblotted with anti-pSmad3 to show the total level of phospho-Smad3. (**g**) Phosphorylation of Akt enhances the Akt–Smad3 interaction. Co-immunoprecipitation studies were performed as described in **a**. AA: T308A-S473A; DD: T308D-S473D. (**h**) Kinase-inactive Akt binds to Smad3. The interaction of Smad3 and Akt mutant in 293T cells was measured as described in **a**.

tion sites (the AA mutant; Fig. 1g) consistently weakened the Akt–Smad3 interaction, whereas those that mimic phosphorylation (DD) enhanced it. Although experiments with recombinant Akt (Fig. 1c) suggest that phosphorylation of Akt is not absolutely necessary for interaction with Smad3, it may enhance this interaction in cells.

Because TGF-β induces apoptosis and disrupts the Smad3–Akt complex, we reasoned that the Smad3–Akt interaction promotes survival. This could occur either through inhibition of Smad3 by Akt, or activation of Akt by Smad3. The latter possibility is unlikely because Smad3-associated Akt had a similar kinase activity as free Akt (data not shown) and because overexpression of Smad3 promoted TGF-β-induced apoptosis (Fig. 4c). To test whether Akt inhibits Smad3, we examined the effect of Akt expression on Smad3-induced transcriptional activation. As reported previously, overexpression of Smad3 activated transcription even in the absence of TGF-β (Fig. 2a). Expression of either wild-type Akt (Fig. 2b) or a constitutively active Akt (myr-Akt; Fig. 2a) inhibited this activation. As Akt did not alter the expression level of Smad3 (Fig. 2a, b), Akt most probably attenuated the transcription activity of Smad3. This inhibition requires phosphorylation of Akt, as the AA mutant failed to block Smad3-mediated transcription but the DD mutant readily did so (Fig. 2b). As a result of a reduced binding affinity for Smad3-3D, Akt did not inhibit Smad3-3D-induced transactivation (see Supplementary Information, Fig. S1a). In addition, Akt did not affect transcription of bone morphogenic (BMP)-responsive promoters (see Supplementary Information, Fig. S1b). Finally, Akt also inhibited transactivation of a GAL4 DNA-binding-site-containing promoter by the GAL4–Smad3 fusion protein (see Supplementary Information, Fig. S1c). Thus, Akt specifically and directly inhibits the transcription activity of Smad3.

The human Hep3B line undergoes apoptosis in response to TGF-β (Fig. 4a) in a Smad3-dependent manner[12]. In contrast to the parental Hep3B cells, those stably expressing myr-Akt (Fig. 2c, left panel) or wild-type Akt (Fig. 2d) failed to undergo TGF-β-induced apoptosis[17], indicating that Akt also inhibits the apoptotic activity of Smad3. This inhibition is specific for the apoptotic pathway because these cells still underwent growth arrest (Fig. 2c, right), upregulated the cyclin-dependent kinase (cdk) inhibitor p21CIP1 and downregulated c-myc expression (see Supplementary Information, Fig. S1d) in response to

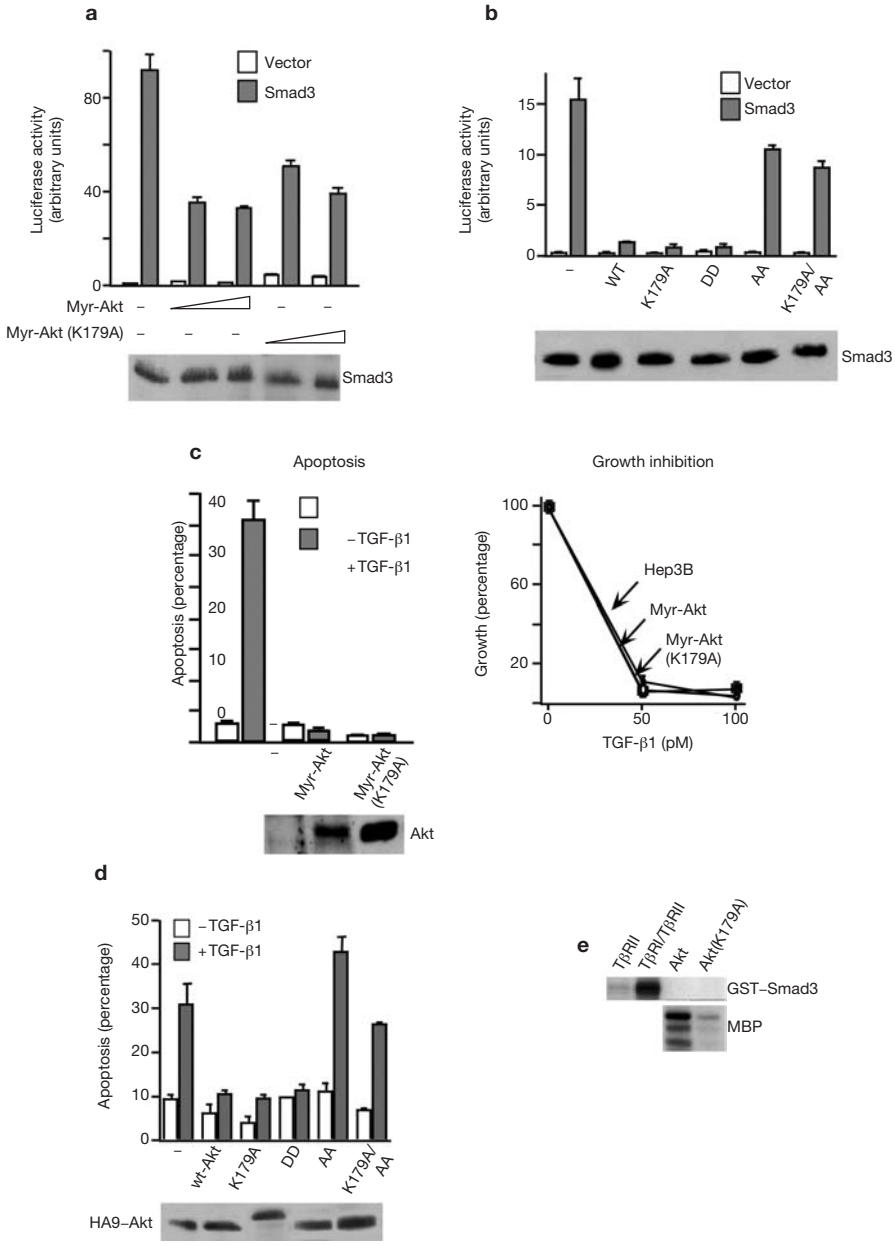

Figure 2 Akt inhibits Smad3 signalling. (**a**) Myr-Akt and myr-Akt(K179A) inhibit the ability of Smad3 to activate transcription. Hep3B cells were transfected with p3TPlux, Smad3 and Smad4 and increasing amounts of the Akt construct. Luciferase activity was measured 48 h later after overnight serum starvation. Expression of Flag–Smad3 in each sample was determined by western blotting of total cell lysate with anti-Flag. (**b**) Phosphorylation of Akt is important for inhibition of Smad3-mediated transcriptional activation. The indicated Akt constructs were co-transfected with Flag–Smad3 and p3TPlux into Hep3B cells. Luciferase assay and western blotting were performed as in **a**, except that cells were not serum starved. (**c**) Myr-Akt and myr-Akt(K179A) block TGF-β-induced apoptosis but do not affect growth arrest. Untransfected Hep3B cells or Hep3B cells stably transfected with myristylated Akt (myr-Akt) or myr-Akt(K179A) were treated with TGF-β1 for 48 h. Apoptosis was assessed by flow cytometry. For growth arrest, cells were plated in triplicate in the presence of indicated concentrations of TGF-β. Cell numbers were counted after 4 days. Expression levels of Akt were measured by western blotting with anti-Akt. (**d**) Phosphorylation of Akt is required for the protection of Hep3B cells from TGF-β-induced apoptosis. Parental Hep3B or Hep3B cells stably expressing the indicated Akt construct were stimulated with TGF-β for 48h. Apoptosis assay was performed as in **c**. Expression of Akt constructs was determined by western blotting of cell lysates with anti-HA. (**e**) *In vitro* kinase assay. Type II (TβRII) and type I (TβRI) TGF-β receptor kinases, and wild-type or K179A Akt were isolated from transfected 293T cells by immunoprecipitation and incubated with GST–Smad3 or MBP in the presence of γ-^{32}P-ATP. ^{32}P-labelled GST–Smad3 or MBP were resolved by SDS–PAGE.

TGF-β, probably because the growth-inhibitory response can also be mediated by Smad2. Consistent with the requirement for Akt phosphorylation in binding to Smad3, the stably expressed AA mutant did not protect cells from apoptosis, whereas the DD mutant did so efficiently (Fig. 2d).

Akt could inhibit Smad3 by directly phosphorylating it or by physi-

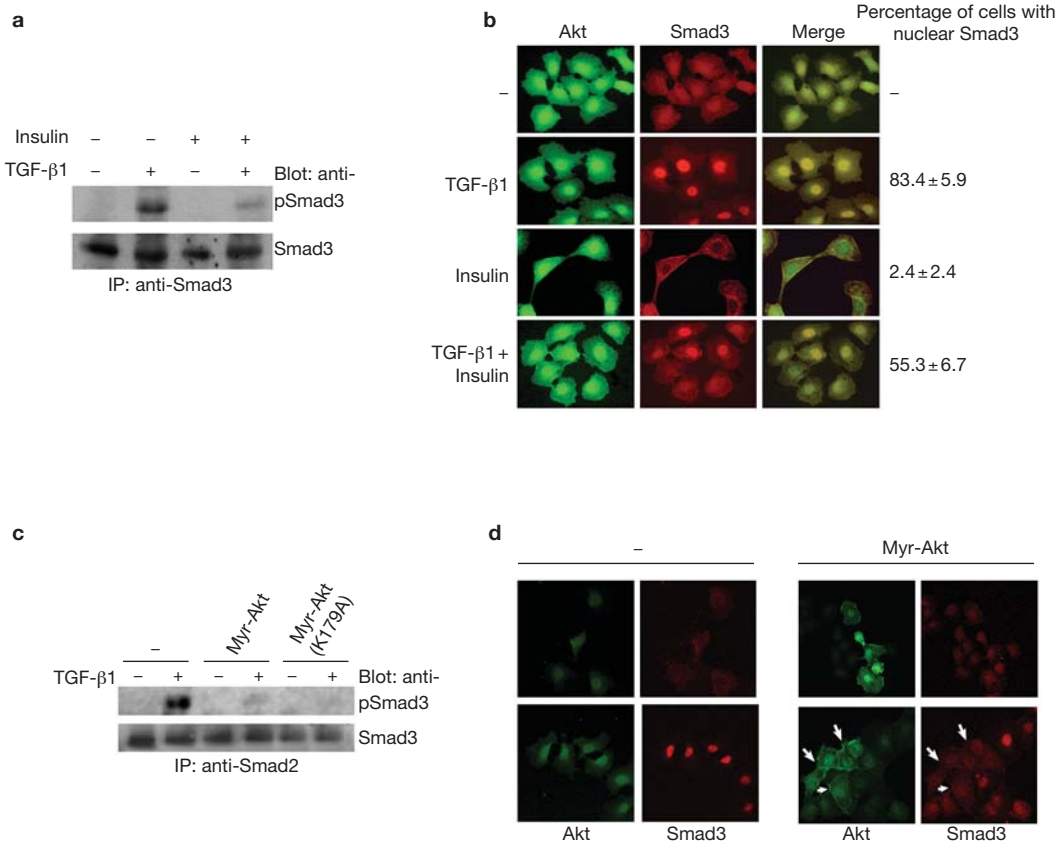

Figure 3 Akt sequesters Smad3 to prevent its phosphorylation and nuclear translocation. (**a**) Insulin attenuates TGF-β-induced phosphorylation of Smad3. Ba/F3 cells were treated for 1 h with TGF-β1, insulin, or both. Phosphorylation of Smad3 was detected by western blotting of anti-Smad3 immunoprecipitates with anti-phospho-Smad3 (top). Total levels of Smad3 in the immunoprecipitates were detected by western blotting with anti-Smad3 (bottom). (**b**) Insulin attenuates TGF-β-induced nuclear translocation of Smad3. RIE-1/FSmad3 cells were treated with TGF-β, insulin, or both, as indicated. Cells were fixed and stained with anti-Akt or anti-Flag (for Smad3) followed by Alexa 488-conjugated goat anti-rabbit and Alexa 594-conjugated goat anti-mouse, and examined by fluorescence microscopy. Quantification of Smad3 nuclear translocation is shown on the right, and the data were obtained from three independent experiments. (**c**) Myr-Akt and myr-Akt(K179A) attenuate TGF-β-induced phosphorylation of Smad3. Hep3B cells, or those stably expressing myr-Akt or myr-Akt(K179A), were stimulated with TGF-β1 for 1h, and phosphorylation of Smad3 was measured by western blotting of anti-Smad3 immunoprecipitates with anti-pSmad3. (**d**) Myr-Akt prevents nuclear localization of Smad3 in response to TGF-β. RIE-1/FSmad3 cells or RIE-1/FSmad3/myr-Akt cells were treated as indicated, stained as in **b** (except that rhodamine-conjugated goat anti-mouse was used), and examined by confocal microscopy. Note that the anti-Akt stain includes both endogenous Akt and the introduced myr-Akt. RIE/FSmad3/myr-Akt cells were from a pool of myr-Akt-expressing cells. Cells with a higher level of myr-Akt expression in this pool are indicated by arrows.

cally sequestering Smad3 to prevent its activation., Unlike the activated TGF-β receptors, Akt did not phosphorylate Smad3 in an *in vitro* kinase assay, even though this Akt kinase was active and phosphorylated myelin basic protein (MBP) efficiently (Fig. 2e). Thus, Akt cannot directly phosphorylate Smad3. Furthermore, the kinase-inactive mutants, myr-Akt(K179A) or Akt(K179A), which were inactive in substrate phosphorylation (Fig. 2e and data not shown), inhibited Smad3-induced transcription and protected Hep3B cells from TGF-β-induced apoptosis to the same extent as myr-Akt or wild-type Akt (Fig. 2a–d). In addition, Akt(K179A) interacted with Smad3 as efficiently as wild-type Akt (Fig. 1h). Thus, the kinase activity of Akt is dispensable for inhibition of Smad3 signalling, and Akt does not block TGF-β-induced apoptosis through direct phosphorylation of either Smad3 or a downstream target[21–24].

We then examined whether activation of Akt affects TGF-β-induced phosphorylation of Smad3 using a phospho-Smad3-specific antibody[25]. As shown previously, TGF-β induced a marked increase in the phosphorylation of Smad3 (Fig. 3a). This increase was reduced after insulin stimulation (Fig. 3a), indicating that activation of Akt can attenuate receptor-mediated phosphorylation of Smad3. A similar inhibition of Smad3 phosphorylation has been observed with IRS-1 (ref. 26).

Next, we examined the effect of Akt activation on nuclear translocation of Smad3 in RIE-1/FSmad3 cells stably expressing Flag–Smad3. Because Smad2 and Smad3 are highly homologous, Flag–Smad3 allows specific localization of Smad3. In the absence of growth-factor stimulation, Akt was localized throughout the cells whereas Smad3 was predominantly cytoplasmic (Fig. 3b). TGF-β induced nuclear translocation of Smad3 without affecting the localization of Akt. Insulin stimulated translocation of Akt to the cell membrane. Notably, it also induced a strong membrane staining of Smad3 that co-localized with Akt, and an exclusion of Smad3 from the nucleus. In cells exposed to both TGF-β and insulin, Smad3 displayed a reduced ability to accumulate in the nucleus. Thus, activation of Akt results in sequestration of

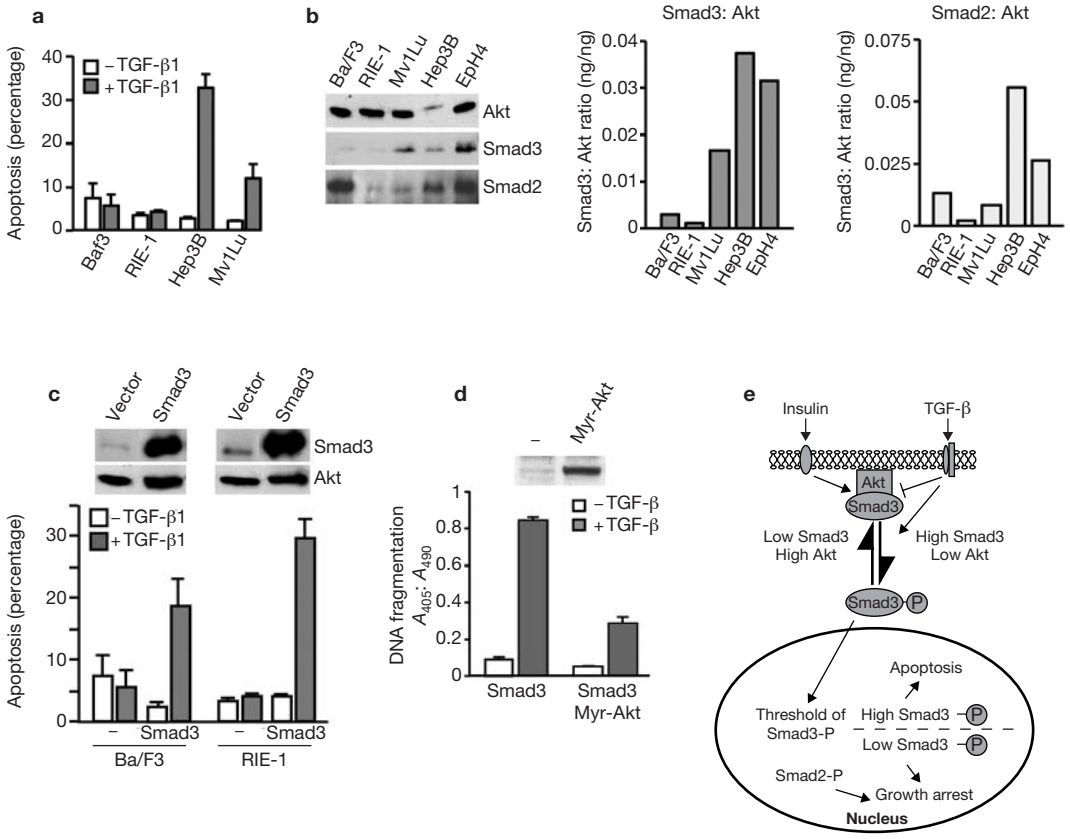

Figure 4 The ratio of Smad3 to Akt determines the sensitivity to TGF-β-induced apoptosis. (**a**) Different cell lines display varying sensitivity to TGF-β induced apoptosis. The indicated cell lines were treated with TGF-β1 for 48 h, and apoptosis was quantified by flow cytometric measurement of DNA content. (**b**) The Smad3:Akt ratio correlates with sensitivity to TGF-β-induced apoptosis. Equal amounts of total cellular proteins from various cell lines were subjected to western blotting with anti-Akt or anti-Smad2/3 (left). Density of the bands in the western blots was quantified and normalized to the intensities of known amounts of recombinant Akt or Smad3 on the same blot to measure actual amounts (in ng) of Akt, Smad3, and Smad2 present in the lysates. Ratios of Smad3:Akt and Smad2:Akt are shown in the right panels. (**c**) Increasing the Smad3:Akt ratio by overexpressing Smad3 sensitizes cells to TGF-β-induced apoptosis. Ba/F3 and RIE-1 cells stably expressing Flag–Smad3 were stimulated with TGF-β1 for 48 h, and their ability to undergo apoptosis was determined by flow cytometry (Ba/F3) or soluble histone ELISA assay (RIE-1). (**d**) Myr-Akt represses the apoptotic activity of Smad3. Myr-Akt was stably introduced into the RIE-1/FSmad3 cells. Apoptosis in the presence of TGF-β1 and 0.5% serum was measured by a soluble histone ELISA assay. (**e**) A model for the regulation of Smad3-mediated apoptosis by Akt.

Smad3 at the plasma membrane and in the cytoplasm.

To directly demonstrate that Akt itself sequesters Smad3 at the cell membrane, we compared TGF-β-induced phosphorylation and localization of Smad3 in parental Hep3B cells and in cells stably expressing myr-Akt. Expression of myr-Akt or myr-Akt(K179A) markedly attenuated the phosphorylation and nuclear translocation of Smad3 (Fig. 3c, d, and data not shown). In the pool of myr-Akt-expressing cells in particular, those with a high level of myr-Akt (Fig. 3d, arrows) did not exhibit nuclear translocation of Smad3 after TGF-β stimulation. Thus, Akt physically sequesters Smad3 to attenuate its phosphorylation and nuclear translocation in a kinase-independent manner.

This model predicts that the relative amounts of Akt and Smad3 may regulate the sensitivity of cells to TGF-β-induced apoptosis. Indeed, in Hep3B and EpH4 cells (which undergo apoptosis in response to TGF-β), a higher Smad3:Akt ratio was detected, whereas RIE-1, Ba/F3 and HaCat cells (which show only cell-cycle arrest) expressed much less Smad3 relative to Akt (Fig. 4a, b, and data not shown). The Mv1Lu cell line responded to TGF-β treatment by cell-cycle arrest and a low level of apoptosis (Fig. 4a), and showed an intermediate Smad3:Akt ratio (Fig. 4b). The expression level of Akt or Smad3 alone or the Smad2:Akt ratio in these cell lines did not correlate with their apoptotic activity (Fig. 4b).

We further investigated whether changing the ratio of Smad3 to Akt affects the sensitivity of cells to TGF-β-induced apoptosis. Increasing the ratio in Ba/F3 and RIE-1 cells by overexpressing Smad3 rendered these cells sensitive to TGF-β-induced apoptosis (Fig. 4c), and this increased sensitivity was further enhanced by serum deprivation or pretreatment with the PI(3)K inhibitor LY294002 (data not shown) and reversed by co-expression of myr-Akt (Fig. 4d). This response is specific for Smad3, as overexpression of Smad2 or Smad4 did not activate TGF-β-induced apoptosis (see Supplementary Information, Fig. S2a). Similarly, decreasing the Smad3:Akt ratio in Hep3B cells by overexpression of Akt or myr-Akt protected the cells from TGF-β-induced apoptosis[17] (Fig. 2b). This protective effect is specific for the TGF-β pathway, as the cells still underwent apoptosis in response to tumour necrosis factor-α (TNF-α; see Supplementary Information, Fig. S2b).

Our data suggest a mechanism by which Akt promotes survival in a kinase-independent manner (Fig. 4e). Akt directly interacts with and sequesters unphosphorylated Smad3 at the cell membrane and in the cytoplasm. In the absence of growth factors, unphosphorylated Akt

binds to Smad3 with a low affinity, and this complex can be dissociated readily by TGF-β, allowing for activation of Smad3 and downstream signalling. In the presence of insulin or serum, activated Akt forms a higher affinity complex with Smad3 that is resistant to dissociation by TGF-β (Fig. 1d and data not shown), resulting in sequestration of Smad3 outside the nucleus and preventing its activation. Initiation of TGF-β-induced apoptosis may require a threshold level of nuclear Smad3. In cells with a high Smad3:Akt ratio, enough Smad3 translocates to the nucleus to initiate apoptosis. If the ratio of Smad3:Akt favours Akt instead, Akt reduces the amount of nuclear Smad3 through sequestration to a level below the required threshold, thereby inactivating the apoptotic and possibly other Smad3-specific responses. However, these cells are still able to undergo growth inhibition because of the presence of Smad2. Attenuation of Smad3 activity by Akt not only affects apoptosis, but may also have an important role in modulation of other Smad3-mediated processes, such as anti-inflammatory responses[27,28]. Thus, our work may have broad implications in many pathological conditions, as well as in normal developmental processes. □

METHODS

Transfection and generation of stable cell lines. All transfections were performed using Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Hep3B cell lines stably expressing myr-Akt and myr-Akt(K179A) were obtained by transfection with pcDNA3.1 containing the appropriate gene and isolating stable clones after 1–2 weeks of selection in G418. Ba/F3 and RIE-1 cells stably expressing Flag–Smad2, Flag–Smad3 or Flag–Smad4 were generated by infection with retroviruses followed by selection in puromycin, as described previously[29]. RIE-1/FSmad3 cells stably expressing myr-Akt were generated by infection with retroviruses containing pLXSN-myr-Akt, followed by selection in G418.

Analysis of apoptosis. After TNF-α (Invitrogen) or TGF-β1 (R&D Sciences) treatment, floating and adhering cells were combined, fixed in 70% ethanol, stained with propidium iodide (Sigma, St Louis, MO) and analysed on a Beckman-Coulter EPICS XL flow cytometer. Measurement of apoptosis by DNA fragmentation was performed using a Cell Death Detection ELISA kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Immunoprecipitation, western blotting and kinase assay. 1×10^8 Ba/F3 cells were serum-starved and treated for 1 h with TGF-β1 (200 pM), insulin (1 μM; Roche), or both. Cells were lysed in lysis buffer (50mM Hepes-KOH at pH 7.8, 0.42 M NaCl, 5 mM EDTA, 1% NP-40, 3 mM DTT, 0.5 mM PMSF, 200 μg ml^{-1} aprotinin and 200 μM NaF) and subjected to immunoprecipitation with anti-Akt1 (C-20) followed by western blotting with anti-Smad3 (FL-425) or anti-Akt1/2 (H-136). The above antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoprecipitation with anti-Flag–agarose was performed as previously described[29]. Anti-phosphoSmad3 was a kind gift of A. Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden).

For the immune complex kinase assay, anti-Akt or anti-TβRI/II immune complex on the beads was incubated with 2 μg of GST–Smad3 or 5 μg of myelin basic protein (MBP, Sigma) in the presence of 10 μCi of γ^{32}P-ATP for 10 min at 30 °C. Phosphorylated proteins were resolved by SDS–PAGE and detected by autoradiography.

***In vitro* binding assay.** Recombinant Smad3 was purified from *E. coli* as a GST fusion protein and cleaved from GST with thrombin. Approximately 5 μg of immobilized GST or GST–Akt proteins was incubated with 2 μg of recombinant Smad3 for 2 h at 4 °C in binding buffer (50 mM Tris at pH 8.0, 150 mM NaCl, 10% glycerol and 1% Triton X-100). Proteins were eluted from glutathione–sepharose using 5 mM glutathione in 50 mM Tris at pH 8.0. Smad3 that associated with GST–Akt was detected by western blotting with anti-Smad3 (FL-425).

Luciferase assays. For the TGF-β response, Hep3B cells were transfected with 0.5 μg p3TPlux, 0.05 μg Flag–Smad3, 0.1 μg Flag–Smad4 and Akt, as indicated. For the BMP response, Hep3B cells were transfected with 0.75 μg 15XGCCG-Luc, 0.25 μg Flag–Smad1, 0.1 μg Flag–Smad4, 0.5 μg constitutively active Alk3 and HA–Akt, as indicated. For assays with the GAL4–Smad3 fusion protein, Hep3B cells were transfected with 0.5 μg pRF-luc, 0.025 μg GAL4–Smad3 and HA–Akt, as indicated. For all assays, luciferase activity was measured 48 h after transfection. Expression of Smad proteins in the samples was measured by western blotting with the appropriate antibody.

Immunofluorescence microscopy. Untreated cells or cells treated for 1 h with TGF-β (200 pM), insulin (10 μM), or both, were fixed, permeablized, and blocked as described[30] before staining with the appropriate antibodies. Alexa fluor-conjugated secondary antibodies were from Molecular Probes (Eugene, OR). Rhodamine-conjugated goat anti-mouse was from ICN (Aurora, OH). Images were obtained using a Zeiss Axiophot fluorescence microscope and a QImaging digital camera or a Zeiss LSM 510 confocal microscope (Zeiss, Thornwood, NY).

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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